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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

David W. SCOTT and
Elias T. ZAMBIDIS

Serial No.: 09/160,076

Filing Date: September 24 1998

For: TOLEROGENIC FUSION PROTEINS
OF IMMUNOGLOBULINS AND
METHODS FOR INDUCING AND
MAINTAINING TOLERANCE

Examiner: M. Wilson
Group Art Unit: 1633

DECLARATION UNDER 37 C.F.R. § 1.132

I, David W. Scott, one of the co-inventors of the invention described in the above identified patent application, declare and affirm that:

1. I am currently Head of the Immunology Department at the Jerome Holland Laboratories of the American Red Cross, Rockville, Maryland. I am also Professor and Chair of the Department of Immunology at the George Washington University Medical Center. I have been actively involved in the study of basic immunology, with particular emphasis on immunological tolerance, since 1966. I have published numerous research papers, review articles and book chapters on this subject. A curriculum vitae, including a selected list of publications, is attached hereto as Exhibit A.

2. I am an inventor on the above-referenced patent application. In addition to the experimental data presented in the specification, additional experiments have been performed in accordance with the teachings of the specification, using a variety of different antigenic polypeptides in fusion proteins. These experiments (described in detail below) have demonstrated that the expression vectors of the invention allow for induction of tolerance to many different antigens, including antigens involved in

autoimmune disorders, and that tolerance can be induced using either an entire antigenic polypeptide or portion thereof in the fusion protein.

3. In addition to the fusion protein comprising residues 12-26 of the bacteriophage λ c1 protein described in Examples I-V of the instant specification (at pages 31-43), fusion proteins comprising the following six antigenic polypeptides (or portion thereof) have been prepared and studied:

- (i) myelin basic protein - full-length protein;
- (ii) glutamic acid decarboxylase (GAD) - full-length protein;
- (iii) insulin B chain - residues 9-23;
- (iv) interreceptor retinal binding protein (IRBP) - residues 161-180;
- (v) bacteriophage λ c1 protein - full-length protein; and
- (vi) ovalbumin - full-length protein.

Each of these antigenic polypeptides, or portion thereof, was incorporated into an IgG fusion protein according to the teachings of the specification and the IgG fusion proteins were expressed on lymphoid or haemopoietic cells, also in accordance with the teachings of the specification. Cells expressing the IgG fusion proteins were then used in tolerance induction studies in mammalian hosts. As described in further detail below, tolerance was induced using fusion proteins comprising each of the six antigenic polypeptides or portion thereof listed above, thereby demonstrating the general applicability of the expression vectors of the invention in inducing tolerance to a wide variety of different antigens, even when the major epitopes for a given MHC haplotype are not known.

4. For the study of myelin basic protein (MBP), the experimental allergic encephalitis (EAE) mouse model was used. The EAE model is a well established, art-recognized system for studying the autoimmune reaction to myelin basic protein, MBP, as a model for multiple sclerosis. An MBP-IgG fusion retroviral vector construct was prepared using the full-length MBP nucleotide sequence in the construct. Splenic B cell blasts were infected with the retrovirus encoding the IgG fusion protein. An ovalbumin-

IgG (OVA-IgG) fusion construct was used as a control. After expansion of the cells, they were injected intravenously into syngeneic recipients after passive transfer of MBP-specific T cells into the recipients. Mice were followed daily for signs of paralysis (as an indicator of an immune response to MBP) until moribund/sacrifice and their average EAE score (on a scale of 1-5) was assessed. As shown in Figure 1, attached hereto as Exhibit B, mice that were treated with the control OVA-IgG expressing cells had EAE scores of at least 3 within first five days of treatment and reached EAE scores of 5 by day 10. In contrast, mice that were treated with the MBP-IgG expressing cells had EAE scores of only 1-2 over the course of 20 days, indicating that the MBP-IgG construct was effective in inducing tolerance in the MBP-specific T cells that had been transferred into the recipients.

5. For the study of glutamic acid decarboxylase (GAD) and insulin receptor B chain residues 9-23, the NOD mouse model was used. The NOD mouse model is a well established, art-recognized system for studying autoimmune diabetes. A GAD-IgG fusion retroviral vector construct and an insulin B chain 9-23 IgG fusion retroviral vector construct (B9-23-IgG) were prepared using a nucleotide sequence encoding the full-length GAD and a nucleotide sequence encoding residues 9-23 of insulin B chain, respectively, in the constructs. Splenic B cell blasts from NOD mice were infected with the retrovirus encoding either the GAD-IgG or B9-23-IgG fusion protein. A lambda repressor cI 1-102 immunoglobulin fusion protein (1-102-IgG) construct was used as a control. The transduced NOD B cell blasts were transferred to NOD recipients intraperitoneally at 10 weeks (by which time the NOD mice exhibit signs of early diabetes). Mice were followed weekly for glucose levels until moribund. Figure 2, attached hereto as Exhibit C, is a graph showing the percentage of mice exhibiting diabetes from 10 weeks to 19 weeks, wherein the mice were either untreated (squares), treated with the control lambda cI 1-102-IgG construct (circles), treated with the GAD-IgG construct (triangles) or treated with the B9-23-IgG construct (diamonds). The results demonstrated that a lower percentage of mice treated with either the GAD-IgG or B9-23-IgG construct exhibited diabetes over the course of the experiment. Importantly, it was found that a single treatment with the GAD-IgG vector led to a significant delay in the

onset of diabetes, as measured by glucose levels and prolongation of life. In addition, a single treatment with either GAD-IgG or insulin B9-23-IgG vectors after clinical signs of diabetes (week 10) showed significant efficacy. Furthermore, in one experiment, treatment at 17 weeks of age showed modest modulation of disease with either or both vectors, whereas mice treated with the control vector (1-102-IgG) progressed like untreated animals. Analysis of islet pathology in GAD-IgG or B9-23-IgG treated mice suggested that remaining islet survival was maintained, despite inflammation in surrounding areas. Importantly, these data show that NOD B cells can be tolerogenic antigen presenting cells (APC). Thus, these experiments demonstrate that the GAD-IgG and B9-23-IgG constructs were effective in inducing tolerance in an animal model of autoimmune diabetes.

6. For the study of interreceptor retinal binding protein (IRBP), the experimental autoimmune uveitis mouse model was used. The uveitis model is a well established model for studying the autoimmune reaction to IRBP. An IRBP-IgG fusion retroviral vector construct was prepared using the nucleotide sequence of residues 161-180 in the construct, since residues 161-180 had previously been established as a uveitogenic peptide. Silver et al., *Invest. Ophthalmol. Vis. Sci.* (1995), 36:946-954. Splenic B cell blasts were infected with the retrovirus encoding the IgG fusion protein and used to treat unprimed mice challenged with p161-180 from human IRBP in complete Freund's adjuvant to induce uveitis. To test the efficacy of already immune animals, seven-day primed mice received similarly transduced B cell blasts. The results of these experiments are described in detail in Agarwal, R.K. et al., *J. Clin. Invest.* (2000), 106:245-252, a copy of which is attached hereto as Exhibit D. The results demonstrated that a single infusion of transduced cells, 10 days before uveitogenic challenge, protected mice from clinical disease induced with the epitope or with the native IRBP protein. Furthermore, the treatment was protective when initiated 7 days after uveitogenic immunization or concurrently with adoptive transfer of primed uveitogenic T cells. Thus, these experiments demonstrate that the IRBP 161-180-IgG construct was effective in inducing tolerance in an animal model of autoimmune uveitis.

7. For the study of induction of hyporesponsiveness to intact foreign protein, an immunoglobulin fusion retroviral vector construct was prepared using the full-length nucleotide sequence of bacteriophage lambda repressor cI protein (encoding residues 1-102). Bone marrow cells or peripheral B cells were infected with the retrovirus encoding the IgG fusion protein and the cells were adoptively transferred into syngeneic mice to test for tolerance. The results of these experiments are described in detail in Kang et al., *Proc. Natl. Acad. Sci. USA* (1999), 96:8609-8614, a copy of which is attached hereto as Exhibit E. The results demonstrate that when the mice were challenged with p1-102, they failed to respond as effectively as mice treated with mock-transfected control cells to the major epitopes of p1-102 recognized by mice of the haplotypes used in the study. The results of these experiments show that retrovirally mediated transfer of a gene encoding a full-length protein fused to IgG is an effective approach for the purpose of inducing tolerance to all known epitopes of an antigen.

8. In addition to the full-length lambda repressor cI protein described in paragraph 7, we have engineered a fusion protein construct that contains a full-length ovalbumin protein. Cells transduced with this OVA-IgG construct were effective in inducing tolerance to ovalbumin (data not shown), similar to the results for the other experimental systems described herein.

9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

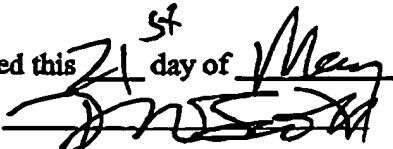
Executed this 21st day of May, 2001

David W. Scott

EXHIBIT A

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME David W. Scott		POSITION TITLE Head, Department of Immunology	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Antioch College, Yellow Springs, OH	Left after 3 years w/o degree to begin grad work		
University of Chicago, Chicago, IL	M.S.	1964	Microbiology
Yale University, New Haven, CT	Ph.D.	1969	Immunology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Experience:

1969 Postdoctoral Fellow, Yale University, Department of Pathology (R.K. Gershon)
 1969-1970 Postdoctoral Fellow, Oxford University, England (J.L. Gowans) *Jane Coffin Childs Fellow*
 1971-1974 Assistant Professor of Immunology, Dept. of Microbiology & Immunology, Duke Univ. Medical Center
 1974-1979 Associate Professor of Immunology, Duke University Medical Center (tenured)
 1975 Visiting Scientist, University of Alberta, Department of Immunology (E. Diener, June-Aug)
 1976-1977 Sabbatical Visitor at Walter and Eliza Hall Institute, Melbourne, Australia (G.J.V. Nossal) *Eleanor Roosevelt Fellowship*
 1979-1983 Professor, Department of Microbiology & Immunology, Duke University Medical Center
 1981 Visiting Scientist, Scripps Clinical & Res. Foundation, La Jolla, CA (N. Klinman, June-July)
 1983-1994 Dean's Professor of Immunology and Head, Immunology Division, Cancer Center and Professor of Microbiology and Immunology, University of Rochester, Rochester, NY
 1986 Visiting Scientist, National Institute for Medical Research, London, U.K. (G.G.B. Klaus, May-December) *Eleanor Roosevelt Fellowship*
 1993 Visiting Scientist, Max-Planck Institute, Freiburg, Germany (G. Köhler, June-August)
 1994- Head, Department of Immunology, American Red Cross, Holland Laboratory, Rockville, MD and Professor of Microbiology and Immunology, George Washington Univ. Medical Center (1998)
 Director, Immunology Training Program, 1999.
 1996- Adjunct Professor of Microbiology and Immunology, Georgetown Univ. Medical Center

Honors and Awards:

Jane Coffin Childs Postdoctoral Trainee, Oxford, England (1969-70)
 Research Career Development Awardee, NIH (1975-80)
 Eleanor Roosevelt Fellow, International Union Against Cancer (1976-77; 1986)
 Dean's Professor of Immunology, University of Rochester Medical Ctr. (1983-1994)

Professional Activities:

NIH Study Section Member, IMB, 1989, 1995, 1997, 1998; AIDS (ARR-1), 1990; IMS-4, 1995;
 DAIDS Panel, Innovative Vaccines, 1998.
 American Cancer Society Advisory Committee, Immunol. & Immunotherapy, 1982-86,
 Vice-Chairman, 1984, Chairman, 1985-86. Council Member, Fall 1990
 Associate Editor, *Journal of Immunology*, 1980-84; Section Editor, 1993-1997
 Associate Editor, *Cancer Immunology and Immunotherapy*, 1984-88
 Editorial Board, *Cellular Immunology*, 1984-present
 Education Committee, AAI, 1980-85, 1993-present; Chairman, 1984-84
 FASEB Education Committee, 1981-90; Chairman 1987-90; IUIS Educat. Comm., 1986-1989
 American Society for Microbiology, Chairman, Pre-College Education Committee, 1990-1994
 Acting Director, Immunol. Allergic & Immunol. Diseases Prgm, NIAID, NIH, summer 1988
 Board Member, Triangle Coalition for Science and Technology Education, 1994-1997
 Advisory Board, Education Development Center, 1984.
 Council, Midwinter Conference of Immunologists, 1997-present.

Research projects ongoing or completed (last three years):**RO1 CA55644-06 (Scott, D.W.) NIH, NCI*****Regulation of B-Lymphoma Growth and Apoptosis***

The major goal of this project is to understand the mechanisms of anti-IgM induced murine B-lymphoma growth arrest and apoptosis, especially with respect to initial signaling, myc and cyclin kinase activity.

PO1 CA78794-01 (Scott, D.W.) NIH, NCI (pending)***Signals Regulating Fas-Mediated Apoptosis in B-cell lines***

This project is examining the regulation of Fas-mediated apoptosis in a series of murine and human B-cell lines. In particular, we are studying the pathways by which anti-Ig crosslinking renders cells Fas-resistant and its role in lymphomagenesis. A second goal is to understand the pathways on natural resistance to Fas-driven apoptosis in a subset of these lines.

RO1 AI35622-01 (Scott, D.W.) NIH, NIAID***Novel Gene Therapy for Tolerance Induction***

The aims of these projects are to establish a bone marrow retroviral transmission approach to autoantigenic epitopes and apply this to a model (EAE) for MS.

#196110 (Scott, D.W.) Juvenile Diabetes Foundation International***Novel Gene Therapies for the Induction of Tolerance in Diabetes***

The aims of this project are to establish a bone marrow retroviral transmission approach to autoantigenic epitopes and apply this to a model of diabetes.

RO1 AI 29691-10 (Scott, D.W.) NIH, NIAID***Regulation of Specific B-Cell Responsiveness to gp120***

The aims of this project are to develop strategies for tolerance to define epitopes in HIV gp120 and apply these to a model of CD4 T-cell apoptosis induced by anti-gp120:gp-120 crosslinking.

Publications (selected from over 170)**David W. Scott, Ph.D.**

Alés-Martínez, J.E., Warner, G.L., and Scott, D.W.: Immunoglobulins D and M mediate signals that are qualitatively different in B cells with an immature phenotype. *Proc. Natl. Acad. Sci. USA* 85: 6919-6923, 1988.

Yao, X.-r. and Scott, D.W.: Expression of protein tyrosine kinases in the Ig complex of anti- μ sensitive and anti- μ resistant B-cell lymphomas: A role of the p55^{blk} kinase in signaling growth arrest and apoptosis. *Immunol. Rev.*, 132: 163-186, 1993.

Scott, D.W. B-cell tolerance in vitro. *Advances in Immunology*, 54: 393-425, 1993.

Fischer, G., Kent, S., Joseph, L., Green, D.R., and Scott, D.W.: Effect of antisense oncogene oligonucleotides on signal transduction in growth inhibitable murine B-cell lymphomas. *J. Exp. Med.*, 179: 221-228, 1994.

Green, D. R. and Scott, D.W. Activation-induced apoptosis in lymphocytes. *Current Opinion in Immunology*, 6: 476-487, 1994.

Joseph, L., Ezhevsky, S., and Scott, D.W. Lymphoma models for B-cell activation and tolerance. XI. Anti-IgM treatment induces growth arrest by preventing the formation of an active kinase complex which phosphorylates pRB in G₁. *Cell Growth and Differentiation*, 6: 51, 1995.

Yao, X.-r., Flaswinkel, H., Reth, M. and Scott, D.W. Ig α or Ig β Cytoplasmic tails containing an Immunoreceptor Tyrosine-based Activation Motif (ITAM) can independently signal for growth arrest and apoptosis in murine B-lymphoma cells. *J. Immunology* 155: 652, 1995.

Ezhevsky, S., Toyoshima, H., Hunter, T. and Scott, D.W. Role of cyclin A and p27 in anti-IgM-induced G₁ growth arrest of murine B-cell lymphomas. *Molecular Biol. Cell*, 7: 553 1996.

Zambidis, E., and Scott, D.W. Epitope-specific tolerance induction with an engineered immunoglobulin. *Proc. Nat. Acad. Sci.* 93: 5019, 1996.

Scott, D.W., Grdina, T. and Shi, Y. T cells commit suicide but B cells are murdered. *J. Immunology*, 156: 2352, 1996.

Scott, D.W., Lamers, M., Köhler, G., Sidman, C., Maddox, B., Wang, R., and Carsetti, R. Regulation of spontaneous and anti-receptor induced apoptosis in adult murine B-cells by c-Myc. *Inter. Immunology*, 8: 1375, 1996.

Zambidis, E., Kurup, A. and Scott, D.W. Genetically-transferred central and peripheral immune tolerance via retroviral-mediated expression of immunogenic epitopes in hematopoietic progenitors or peripheral B lymphocytes. *Molec. Medicine*, 3: 212, 1997.

Zambidis, E., Barth, R. and Scott, D.W. Both resting and activated B lymphocytes expressing engineered peptide-immunoglobulin molecules serve as highly efficient tolerogenic vehicles in immunocompetent adult recipients. *J. Immunology*, 158: 2174, 1997.

Scott, D.W., Brunner, T., Donjerkovic, D., Ezhevsky, S., Grdina, T., Green, D., Shi, Y. and Yao, X.-R. Murder and suicide: A tale of T and B cell apoptosis. In: Programmed Cell Death (ed. by Y. B. Shi, Y. Shi, D.W. Scott and X. Yu), Plenum Press, New York, p. 91, 1997.

Scott, D.W., Donjerkovic, D., Maddox, B., Ezhevsky, S., and Grdina, T. Role of c-myc and p27 in anti-IgM induced B-lymphoma apoptosis. In: Mechanisms in B-cell neoplasia (ed. by M. Potter and F. Melchers) *Contemp. Topics in Immunobiol.* p. 103, 1997.

Kang, Y. and Scott, D.W. An ongoing immune response to HIV envelope glycoprotein in Human CD4 transgenic mice contributes to T cell decline upon intravenous administration of gp120. *Eur. J. Immunol.* 28: 2253-2264, 1998.

Donjerkovic, D., and Scott, D.W. Regulation of p27kip1 accumulation in murine B-lymphoma cells: role of c-myc and calcium. *Cell Growth and Differentiation*, 10: 695-704.

Kang, Y., Melo, M., Deng, E., Tisch, R., El-Amine, M. and Scott, D.W. (1999) Induction of hyporesponsiveness to intact multi-determinant foreign protein via retroviral-mediated gene expression: the IgG scaffold is important for induction and maintenance of immunological hyporesponsiveness. *Proc. Nat. Acad. Sci.*, 96: 8609.

Mueller, C.M., and D.W. Scott. 1999. Differential sensitivity of murine B lymphoma cell lines to ligation of the Fas receptor. Submitted. Under revision.

Mueller, C., J.A. Hinshaw, and D.W. Scott. 1999. B-cell receptor-induced protection from Fas-mediated apoptosis. In preparation

Agarwal, R. K., Kang, Y., Zambidis, E., Scott, D.W., Chan, C. and Caspi, R.R. 1996. Retroviral gene transfer of an immunoglobulin-antigen fusion protein protects from autoimmune disease. Submitted.

Scott, D.W., Donjerković, D., Carey, G., Mueller, C., Liu, S., and Tonnetti, L. B-cell receptor and Fas-mediated signals for life and death. *Immunol. Reviews* 176: in press, 2000.

Donjerkovic, D., Carey, G., Mueller, C., Liu, S., and Scott, D.W. Life and death decisions in B1 lymphomas. In: Mechanisms in B-cell neoplasia: Role of B1 and natural antibody producing cells in B-cell neoplasia (ed. by M. Potter and F. Melchers) *Contemp. Topics in Immunobiol.* In press, 2000.

EXHIBIT B

Figure 1

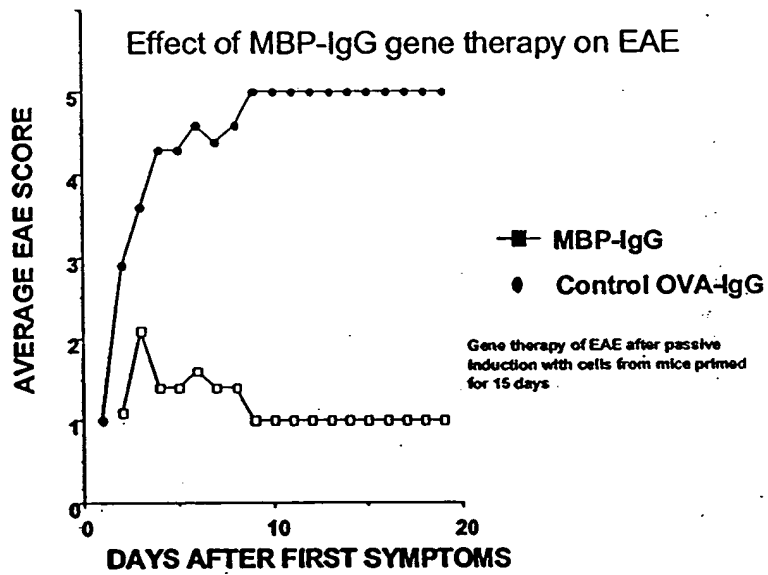


EXHIBIT C

Figure 2

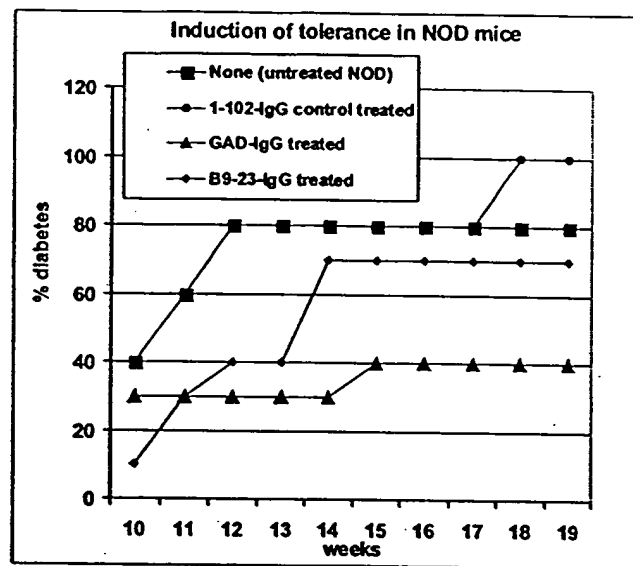


EXHIBIT D

Retroviral gene therapy with an immunoglobulin-antigen fusion construct protects from experimental autoimmune uveitis

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Immunoglobulins can serve as tolerogenic carriers for antigens, and B cells can function as tolerogenic antigen-presenting cells. We used this principle to design a strategy for gene therapy of experimental autoimmune uveitis, a cell-mediated autoimmune disease model for human uveitis induced with the uveitogenic interphotoreceptor retinoid-binding protein (IRBP). A retroviral vector was constructed containing a major uveitogenic IRBP epitope in frame with mouse IgG1 heavy chain. This construct was used to transduce peripheral B cells, which were infused into syngeneic recipients. A single infusion of transduced cells, 10 days before uveitogenic challenge, protected mice from clinical disease induced with the epitope or with the native IRBP protein. Protected mice had reduced antigen-specific responses, but showed no evidence for a classic Th1/Th2 response shift or for generalized anergy. Protection was not transferable, arguing against a mechanism dependent on regulatory cells. Importantly, the treatment was protective when initiated 7 days after uveitogenic immunization or concurrently with adoptive transfer of primed uveitogenic T cells. We suggest that this form of gene therapy can induce epitope-specific protection not only in naive, but also in already primed recipients, thus providing a protocol for treatment of established autoimmunity.

J. Clin. Invest. 106:245–252 (2000).

Introduction

The failure to discriminate between self and nonself leads to clinical manifestations of autoimmunity. A number of experimental procedures have been proposed to induce protective tolerance to autoantigens (1–5); however, tolerogenesis in an already immune host has been difficult to achieve. Based on the tolerogenic properties of immunoglobulin carriers combined with the efficacy of B-cell antigen presentation for unresponsiveness, we demonstrated previously that a retroviral vector encoding an immunodominant peptide of phage λ repressor protein in frame with a murine IgG1 heavy chain was tolerogenic when transduced into bone marrow cells or LPS-stimulated B cells (6). Genetically compatible recipients of the transduced cells were rendered hyporesponsive to the λ repressor epitope.

In the present study, we have built on this model antigen system as the basis of an approach for induction of protective tolerance from autoimmune disease. We used the model of experimental autoimmune uveitis (EAU), a T-cell mediated disease that targets the neural retina. EAU can be induced in susceptible animals by immunization with retinal antigens or their fragments or by adoptive transfer of T cells specific to these antigens (7, 8). The underlying immunopathogenic mechanisms are shared by other cell-mediated autoimmune

diseases, permitting a generalization of therapeutic approaches and conclusions developed in the uveitis model to other systems. Importantly, EAU serves as a model of human autoimmune uveitis, which is estimated to cause 10% of the cases of severe visual impairment. Current treatments for uveitis employ systemic medications that have severe side effects and are globally immunosuppressive (9). Thus, there is an urgent need to develop effective immunotherapeutic strategies that are nontoxic and that specifically target the pathogenic cell population.

To test whether tolerance induction by gene transfer could be used to ameliorate autoimmunity, we engineered a chimeric retrovirus encoding a major pathogenic epitope (residues 161–180 of mouse interphotoreceptor retinoid-binding protein [IRBP]) (10) in frame with mouse IgG1 heavy chain. Recipients of B cells transduced with the chimeric retrovirus and challenged with a uveitogenic regimen of the 161–180 epitope were significantly protected from disease. Most importantly, this gene therapy approach was effective even when initiated 7 days after uveitogenic immunization, when uveitogenic effectors are already primed, although a more intense tolerogenic regimen was required. We suggest that this form of gene therapy can be used to induce epitope-specific protection not only

the naive but also in already primed recipients pointing to a possible clinical applicability of this approach.

Methods

Animals. Female B10.RIII (H-2^b) mice, 6–8 weeks old, were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA) and were housed under pathogen-free conditions. Animal care and use was in compliance with institutional guidelines.

Synthetic peptide. The murine 161-180 peptide (SGIPYVLSYLHPGNTVMHVD) and its human homologue (SGIPYLSYLHPGNTILHVD) were synthesized on a PE Applied Biosystems (Foster City, California, USA) peptide synthesizer as described previously (10).

Retroviral constructs and virus producer cell lines. The MBAE retroviral vector encoding the 12-26 epitope of bacteriophage λ cI repressor protein fused in frame to mouse IgG1 heavy chain and its viral producer cell line (F6P), described previously (6), were used as a mock control in the current study. The IRBP161-180-IgG-MBAE retroviral vector, as well as the high-titer ($\sim 10^6$ neomycin-resistant NIH 3T3 CFU per milliliter) and helper virus-free packaging cell line (#52/139), were similarly generated (ref. 6; Figure 1). Viral producer cell lines were stored in liquid nitrogen and freshly thawed for individual experiments.

Gene transfer into LPS-stimulated B cells for prevention and treatment of EAU. Retroviral-mediated gene transfer into LPS-stimulated (*Escherichia coli* 055:B5; Sigma Chemical Co., St. Louis, Missouri, USA) B cells has been described (6, 11). Briefly, the cells to be transduced (B-cell blasts stimulated with LPS for 24 hours) were cocultured (4×10^6 cells/mL; 6-mL cultures) with irradiated (20 Gy) 52/139 or F6P (mock) virus-producer monolayers in the presence of 6 μ g/mL polybrene and 50 μ g/mL LPS for 24 hours. The transduced B-cell blasts were injected ($30\text{--}40 \times 10^6$ cells/mouse, intraperitoneally) into syngeneic recipients. On the basis of experiments with a green fluorescent protein-containing vector, the percentage of productively transduced cells is estimated to be about 10–20% of the infused inoculum. In a disease-prevention protocol, 8–10 days after receiving the transduced B-cell blasts, the recipient mice were immunized for EAU induction with either the mouse or the human homologue of peptide 161-180 (see below). In a disease-reversal protocol, recipients were given three infusions of transduced B cells on days 7, 9, and 11 after the uveitogenic immunization.

The murine IgG1 heavy chain used in the retroviral vector binds the hapten NIP after assembling with λ light chain (6, 11). Therefore, production of retroviral constructs can be confirmed by infection of J558L myeloma cell line and measurement of NIP-specific IgG1 in the supernatants. ELISA results showed that J558L cells transduced with m161-180-IgG1 and cI 12-26-IgG produced 52 ng/mL and 68 ng/mL chimeric IgG, respectively. Gene expression *in vivo* was verified by the detection of the NIP-specific IgG1 heavy chain in sera of recipient mice (6, 11).

EAU induction and scoring. Mice were immunized subcutaneously with murine peptide (100 μ g) or human peptide (10–25 μ g) in CFA (1:1, vol/vol) supplemented with *Mycobacterium tuberculosis* to 2.5 mg/mL. *Bordetella pertussis* toxin (Sigma Chemical Co.) (PTX; 1.0 μ g in 100 μ L) was given intraperitoneally with the murine homologue only. EAU by adoptive transfer was induced with $20\text{--}40 \times 10^6$ pooled lymph node and spleen cells and cultured for 3 days with the human 161-180 peptide, as described (12). Eyes collected for histopathology 21 or 28 days after immunization (10–12 days after adoptive transfer) were fixed and embedded in methacrylate (13). The incidence and severity of EAU on hematoxylin and eosin-stained sections were scored on an arbitrary scale of 0 to 4, according to a semiquantitative system described earlier (8, 13).

The choice of peptide (human or murine) used for challenge depended on the nature of the experiment. For measuring immunological responses the murine peptide is preferred because of the possibility that the human peptide might elicit clones recognizing heterologous specificities absent from the tolerizing mouse peptide irrelevant to disease, which would confound the readout. For EAU induction the human peptide is usually preferred because it obviates the need to use PTX; clones induced to the human peptide that do not recognize autologous specificities would not come into play in a disease readout system.

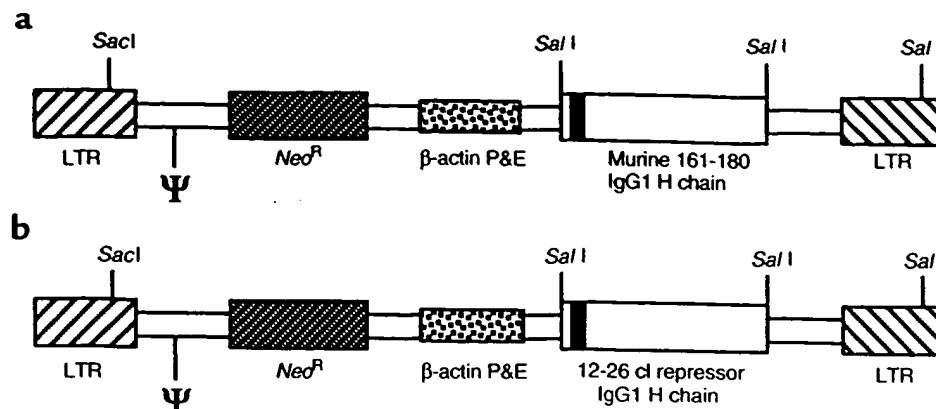
Immunologic assays. Delayed-type hypersensitivity (DTH) was measured on day 21, 48 hours after challenge with 10 μ g of the immunizing peptide (or in some cases of IRBP) into the ear pinna (10). The response is calculated as the difference between thickness in micrometers of the antigen-injected and the PBS-injected ears.

Lymphocyte proliferation. Spleens and draining lymph nodes collected on day 21 after immunization were pooled within groups. Triplicate 0.2-mL cultures of 5×10^5 cells in DMEM (Hyclone Laboratories Inc., Logan, Utah, USA) supplemented as described (10, 12) and containing 1.5% syngeneic mouse serum were incubated in round-bottomed 96-well plates with graded doses of human or murine 161-180 peptide or 1 μ g/mL of PHA (Murex Biotech Ltd., Dartford, United Kingdom). The cultures were pulsed after 48 hours with 1 μ Ci [3 H] thymidine per well for an additional 18 hours and were harvested and counted by standard liquid scintillation.

Determination of cytokine production. Lymph nodes and spleen cells collected 10 or 21 days after immunization were cultured as for the proliferation assay, except that 10^6 cells/200 μ L were plated in flat-bottomed 96-well plates with either peptide (30 μ M) or PHA (1 μ g/mL). Supernatants were collected 24 hours later for IL-2 assay and 48 hours later for all other cytokines. IL-2, IL-4, IL-5, and TNF- α were assayed using ELISA kits from Endogen (Woburn, Massachusetts, USA), and IFN- γ , IL-10, and TGF- β 1 (total) were assayed using ELISA kits from Genzyme Pharmaceuticals (Cambridge, Massachusetts, USA).

Figure 1

Structure of tolerogenic mIRBP161-180-IgG (a) and control cl 12-26-IgG (b) constructs. LTR, long terminal repeat; Ψ , packaging signal; Neo^R , neomycin resistance gene; P&E, promoter and enhancer.



Antibody responses. Peptide-specific IgG2a and IgG1 antibodies were determined in individual sera by ELISA on plates coated with murine or human 161-180 peptide (3 μ g/mL) and probed with horseradish peroxidase-conjugated goat anti-IgG subclass-specific antibodies (Southern Biotechnology Associates, Birmingham, Alabama, USA), as described earlier for another antigen (14). The concentrations of anti-peptide Ab isotypes were determined using standard curves generated by coating the wells with anti-isotype antibodies and adding polyclonal isotype Ig standards.

Adoptive transfer of B cell-depleted splenocytes. B220-positive cells were depleted from spleen cell suspensions by negative selection with anti-B220-coated magnetic beads, per manufacturer's instructions (Dyna, Lake Success, New York, USA). Flow cytometric analysis (FAC-Scalibur; Becton Dickinson Immunocytometry Systems, San Jose, California, USA) of the depleted population after surface staining with fluoresceinated antibodies for CD19 and CD3 confirmed less than 0.5% of residual B cells. Recipient mice were infused intravenously with an equivalent of two donor spleens (approximately 50×10^6 cells) in 0.5 mL and were challenged with antigen 24–72 hours after adoptive transfer.

Statistical analysis, reproducibility, and data presentation. Experiments were repeated at least twice; response patterns were highly reproducible. Statistical analysis of EAU scores was by frequency analysis, using Snedecor and Cochran's test for linear trend in proportions (15). Disease severity for each animal was calculated as average of both eyes. Statistical analysis of immunological responses was by 2-tailed independent *t* test.

Results

Recipients of LPS blasts transduced with antigen-IgG construct are protected from EAU. Zambidis et al. (6) reported that infusion of retrovirally transduced cells containing a construct encoding a dominant epitope of phage λ repressor protein in frame with an IgG heavy chain (cl 12-26-IgG) led to long-term suppression of the immune response to that epitope. To adapt this methodology to immunotherapy of autoimmune disease, the murine 161-180 epitope of IRBP was engineered into the same vector (m161-180-IgG) (Figure 1).

LPS-stimulated B cells prepared from spleens of naive B10.RIII mice were transduced with m161-180-IgG vector or the control cl 12-26-IgG vector. Recipients of 30–40 million transduced cells were challenged after 8–10 days for induction of EAU by immunization with the murine or the human homologue of peptide 161-180 (designated as m161-180 or h161-180, respectively). Note that whereas treatment was always with the autologous murine epitope, uveitogenic challenge was either with the murine or with the human homologue (for reasons stated in Methods). Histopathology of eyes obtained 21 days after immunization, when disease in controls is at its peak, showed that the single infusion of transduced B cells afforded highly significant protection from disease, whether induced by challenge with the autologous murine or the homologous human epitope (Figure 2a). Many of the treated mice remained completely free of disease. Importantly, mice immunized with the native IRBP molecule were also significantly protected, indicating that tolerance to

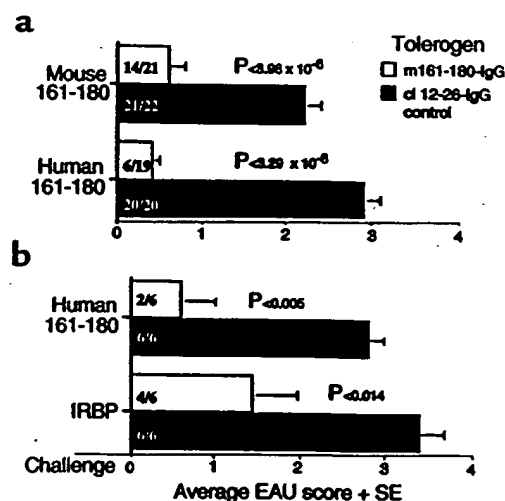


Figure 2

EAU scores in recipients of LPS blasts transduced with mIRBP161-180-IgG, infused 8–10 days before uveitogenic challenge with (a) murine 161-180 or human 161-180, or (b) human peptide or whole IRBP. The incidence (number of positive out of total mice) is shown within the bars. The data were compiled from five experiments.

dominant epitope can afford protection against the whole multiepitope protein (Figure 2b). Typical EAU histopathology in mice that received cells transduced with the tolerogenic versus control vectors is shown in Figure 3. In experiments not shown here, animals challenged with peptide as late as 2 months after the tolerogenic infusion were still protected (data not shown).

To find out whether packaging cells carried over with the transduced B cells might have contributed to the protection, their percentage in the tolerizing inoculum was assessed by staining cytocentrifuged preparations with Giemsa and by performing a differential count of lymphocytes versus fibroblasts. Contamination by packaging cells was found to be 3–5%. Two million

p161-180-IgG retrovirus-producing irradiated packaging cells (equivalent to 6% contamination) infused into recipient mice had no protective effect whatsoever against peptide challenge, confirming that tolerogenesis was caused by the transduced B-cell blasts.

Cell-mediated and humoral responses in treated mice. Mice infused with transduced LPS blasts as above were challenged for DTH with the immunizing peptide on day 19. Mice that were functionally protected from EAU (whose disease scores are shown in Figure 2) had moderately, albeit significantly, reduced DTH responses ($P < 0.002$, data not shown). Draining lymph node and spleen cells collected from these mice on day 21 were tested for proliferation against the immunizing peptides (Figure 4). Mice challenged with the murine peptide had significantly and reproducibly depressed proliferative responses and exhibited a dose-response shift over approximately 1.5 logs of antigen concentration. The effect on *in vitro* proliferation to human peptide was less pronounced, which probably reflects the fact that the tolerizing peptide and the immunizing peptide are not identical (data not shown).

Antigen-specific cytokine responses were examined 10 and 21 days after immunization. We chose to study responses only of mice immunized with the murine 161-180 homologue so that any cross-reactive responses would not obscure specific hyporesponsiveness. Type 1/proinflammatory cytokines IL-2, IFN- γ , and TNF- α and type 2/anti-inflammatory cytokines IL-4, IL-5, IL-10, and TGF β 1 were assayed by ELISA in 48-hour supernatants of cells stimulated with the immunizing peptide. Diminished cytokine responses resembling the effects on DTH and lymphocyte proliferation were seen for IL-2, IFN- γ , and IL-10 (Figure 5). TNF- α secretion by spleen and lymph node cells of the protected group was half that of the control group on day 10 (but not on day 21), and several other cytokines, including TGF- β , did not exhibit consistent differences (data not shown).

Antigen-specific IgG1 and IgG2a antibody responses were assayed in individual sera collected from recipients of protective or mock control cells 21 days after a uveitogenic challenge with mouse 161-180 peptide. Because the switch factor for IgG1 is IL-4 and the switch factor for IgG2a is IFN- γ , these antibody isotypes can also serve as a readout of the Th1 or Th2 bias of the antigen-specific response. Thus, skewing of the IgG1/IgG2a ratio would be indicative of an immune deviation, whereas an overall reduction in both isotypes would indicate a mechanism unrelated to the Th1/Th2 balance. Isotype-specific ELISA showed that, on average, both IgG1 and IgG2a anti-IRBP antibody titers in protected mice were reduced to half the values of controls, although individual titers were variable (Figure 6). Recipients of 161-180-IgG-transduced cells showed a trend toward higher IgG1/IgG2a ratios than the control group. Because of the large individual variability, however, the difference between the group averages did not attain statistical significance (*t* test).

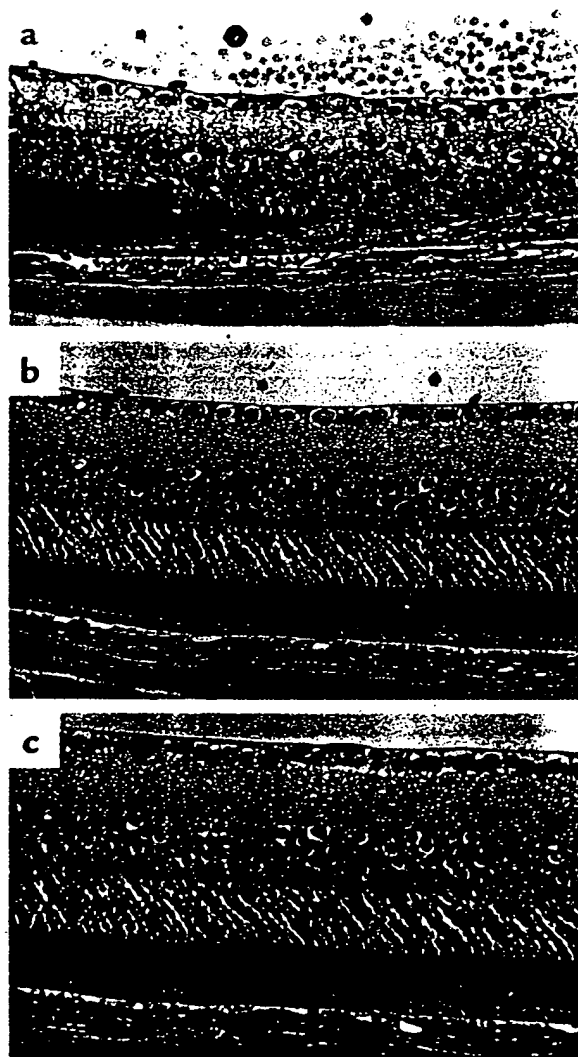
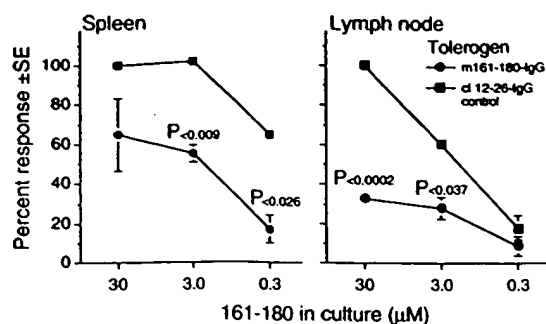


Figure 3

Ocular histology of peptide-immunized mice that had been pretreated with either the LPS-stimulated B cells transduced with the mock control construct (a) or with the tolerogenic construct (b), compared with retina of naive mice (c). Eyes were processed for histology 21 days after uveitogenic immunization. Shown are results after immunization with human peptide. Ocular pathology of mice challenged with the murine construct was essentially identical.

Figure 4

Lymphocyte proliferation to murine 161-180 epitope in protected mice. Shown is an average of two identical experiments. Counts were normalized to mock control at 30 μ M peptide after background subtraction (100%) to compensate for interexperiment variation. (Actual 100% values for spleen and lymph node, respectively, were 31,650 and 42,900 cpm, with background of 7,000 cpm). The EAU scores of these mice are shown in Figure 2.



Protection from EAU is not transferable. We next wished to examine whether spleens of mice that received the protective treatment contain regulatory cells that could adoptively transfer protection to untreated recipients. Donor mice were given LPS-stimulated B cells transduced with the protective or control retroviral construct. After 10 days, when the animals would normally be challenged for EAU induction, their spleens were removed and depleted of B220⁺ cells by immunomagnetic beads (resulting in < 0.5% residual B cells) to minimize carryover of transduced B cells that could be tolerogenic APCs in the adoptive transfer recipient. Recipients infused with an equivalent of two B cell-depleted donor spleens were challenged for EAU development. Recipients of splenocytes from donors who received the protective treatment developed EAU scores equivalent to scores developed by recipients of control splenocytes, suggesting absence of regulatory cells in the transferred population (Figure 7a). Because this interpretation is based on a negative result, a positive control was used to show that a measurable immune function could be successfully transferred under these conditions. In a parallel experiment, an equivalent number of B cell-depleted splenocytes were able to transfer a DTH response from IRBP-immunized donors to naive recipients (Figure 7b).

Induction of protection in primed recipients. For an immunotherapy strategy to be clinically relevant, it must be effective in an already primed subject. To test whether this kind of retroviral gene therapy would be able to ameliorate disease if administered after priming, we infused LPS blasts transduced with the protective or the control constructs into recipients that had been immunized with human 161-180 seven days earlier.

Clinical onset of EAU in this model normally occurs between 9 to 12 days. To confirm that on day 7 the animals have primed cells that are functional in terms of disease, we extracted spleen and lymph node cells from animals immunized with human 161-180 seven days before, and subjected them to a standard 3-day *in vitro* activation before adoptive transfer into naive recipients. Recipients of 40×10^6 cells developed EAU with a mean score of 3, which is directly comparable to scores seen routinely with recipients of an equivalent number of cells extracted 2–3 weeks after immunization.

A single infusion of gene-transduced cells, which was highly effective in protecting preimmune animals, was ineffective in ameliorating disease in primed recipients.

However, three consecutive infusions, given every other day, were highly effective in reducing EAU scores (Figure 8). Furthermore, a single infusion was able to reduce EAU elicited by adoptive transfer of primed T cells. Thus, six of six mice given 30×10^6 m161-180-IgG-transduced B cells and challenged 12 hours later by adoptive transfer of uveitogenic T cells from donors immunized with peptide 161-180 were completely protected. In contrast, three of six recipients of mock control T cells and three of five naive recipients developed EAU. This treatment strategy can, therefore, be protective in a situation where primed effector cells have already been generated.

Discussion

In this study we have used a retroviral gene therapy strategy to prevent or reverse EAU. We demonstrate that recipients of LPS blasts transduced with a tolerogenic construct encoding the murine 161-180 peptide of IRBP in frame with murine IgG1 were highly protected from EAU induced with either the human or the murine uveitogenic peptide homologues and were sig-

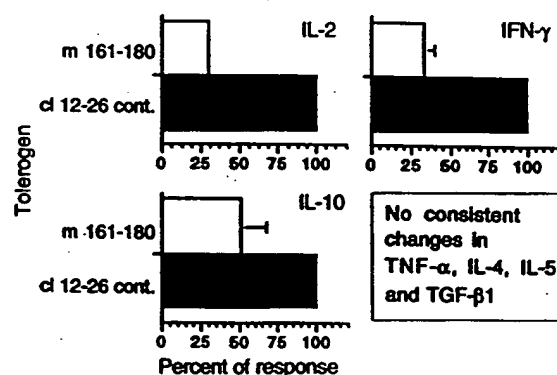


Figure 5

IL-2, IFN- γ , and IL-10 production by spleen cells of tolerized mice to 30 μ M peptide. Shown are IFN- γ and IL-10 production as assayed 21 days after immunization (average of three experiments) and IL-2 as assayed only 10 days after immunization (single experiment). Values are normalized against the mock control (100%). Lower level of detectability in picograms per milliliter was 26 for IL-2, 30 for IFN- γ , and 10 for IL-10. Actual 100% values in picograms per milliliter were 3,600 for IL-2, 910 for IFN- γ , and 56 for IL-10. The pattern of IFN- γ and IL-10 responses on day 10 was the same as on day 21, though the absolute amounts secreted were higher. Lymph node cytokine responses were essentially identical to spleen responses.

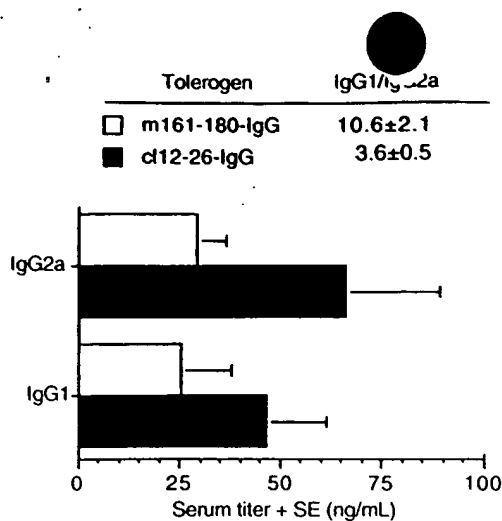


Figure 6
Humoral response to m161-180 in tolerized mice. The bars show average IgG2a and IgG1 titers of 20 mice compiled from four repeat experiments. The IgG1/IgG2a ratio shown at the top was calculated as an average of individual Ig isotype ratios.

nificantly protected from challenge with the whole, multiepitope, IRBP molecule. In experiments not shown here, bone marrow cells transduced with this construct and infused into irradiated recipients had similar protective effects (R.K. Agarwal et al., unpublished observations). It should be pointed out that the term "tolerogenic" is used here to convey the ability to protect from clinical disease, not to indicate lack of an immune response or to imply a particular mechanism.

Whereas the present approach builds on data that demonstrated tolerance induction in a model antigen system of an immunodominant phage λ repressor epitope, it was not a given that such a strategy could be adapted to a self antigen in a therapeutic setting. The cI 12-26 epitope is derived from a nonself antigen, to which the T-cell repertoire is of high affinity. In the case of self epitopes, which have escaped the checkpoint of negative selection in the thymus, the repertoire is of medium and low affinity. There is abundant evidence in the literature that strength of signal determines the quality of the T-cell response to an antigenic stimulus to the point that responses to partial agonists can be totally opposite from responses to full agonists. Our study is therefore an important advance that translates findings obtained in a model antigen system to a therapeutic setting.

It is important to emphasize that the present approach is not restricted to ocular autoimmunity. There are many tissue-specific autoimmune diseases that share essential mechanisms with uveitis (e.g., experimental autoimmune encephalomyelitis [EAE], diabetes, arthritis, and thyroiditis). The therapeutic approach we describe, modified to incorporate the appropriate antigens, is equally applicable to those other disease entities. Recent data indicate that similar constructs containing myelin basic protein (MBP) are able to protect mice from EAE (M. Melo and D.W. Scott, manuscript in preparation). This enhances the therapeutic value of this strategy in cases where a dominant self epitope is known. Thus, the present approach presents a platform that can be generalized for gene therapy of cell-mediated and, potentially, also of antibody-mediated autoimmune diseases.

In a clinical setting, the patient presents in an immunologically primed state, and continuous recruitment of new pathogenic clones is thought to occur in chronic disease. Importantly, the present regimen afforded protection to both unprimed and primed hosts, although the latter may require repeated tolerogenic infusions. In the present experiments we did not address the question of how long the hyporesponsive state persists beyond 2 months; however, in the cI 12-26 model antigen system tolerance lasts at least 4–8 months (Y. Kang, M. El-Amine, and D.W. Scott, manuscript in preparation). We therefore believe that this system may offer a powerful approach in prevention or amelioration of autoimmune disease in a clinical setting.

Previous studies have used intravenous infusions of antigens chemically coupled to autologous immunoglobulins or cells to induce tolerance (1, 3, 16). The advantage of the present approach is that it introduces a self-renewing source of tolerogen that establishes residence in the body and essentially becomes part of "self," as opposed to remaining an exogenous treatment whose effects may be transient. Moreover, in already immune individuals the present approach has an additional advantage over an intravenous bolus of an antigen-Ig conjugate in that it avoids introducing a large amount of antigen into the bloodstream, which might trigger an anaphylactic reaction.

There is also a clear advantage to using ex vivo-transduced autologous cells over direct administration of the chimeric retrovirus, because it largely circumvents the

Figure 7
EAU (a) and DTH (b) scores in recipients of B cell-depleted splenocytes. (a) EAU scores in recipients of cells from donors who received infusion of LPS blasts transduced with the protective or mock control retroviral constructs. Mice were challenged with the human peptide 24 hours after the infusion and scored for EAU on day 21. (b) DTH scores to IRBP in recipients of spleen cells prepared by the same method from IRBP-immunized or from naive donors (positive control). Recipients were challenged with 10 μ g of IRBP into the ear pinna 72 hours after transfer. DTH scores were read after 48 hours.

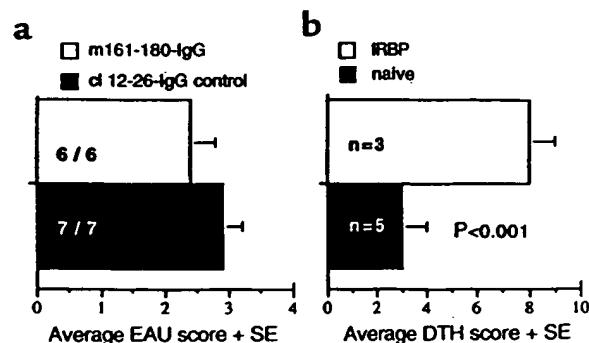
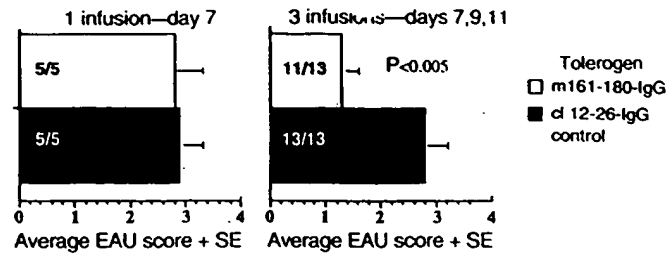


Figure 8

EAU scores in primed recipients of LPS blasts, infused 7 days after uveitogenic challenge with h161-180. The incidence (number of positive out of total mice) is shown within the bars.



problems inherent in administering immunogenic viral vectors in vivo. This may be particularly important in view of the apparent need for repeated tolerogenic treatments in the primed host. It should be noted that protection was obtained in this system with both LPS blasts and with bone marrow cells (R.K. Agarwal et al., unpublished results) as tolerogenic vehicles. Recently McPherson et al. reported protection from EAU in the rat model using reconstitution of irradiated recipients with bone marrow cells transduced with the retinal soluble antigen (17). Because LPS blasts can be used in nonconditioned recipients, they have advantages over bone marrow gene-transfer protocols because of their simplicity and because there is no requirement for myeloablation.

In the current study, a single immunodominant uveitogenic peptide was engineered in frame with the IgG scaffold. Whereas this approach provides an important proof of the concept and, at least in the case of IRBP, also affords significant protection against the whole IRBP molecule, in the future full-length proteins or major domains of autoimmunogenic proteins will need to be employed. In human uveitis, as in other autoimmune diseases, a number of MHC haplotypes have been connected to susceptibility (9, 18) and disease-relevant epitopes recognized by different haplotypes are unknown. Moreover, the now well-recognized phenomenon of epitope spreading (19-21) would require covering of multiple epitopes even within the same haplotype. Data in the phage λ repressor model system indicates that the full-length protein induces epitope-specific tolerance in diverse H-2 haplotypes (11). Thus, using a full-length autoantigen would have the advantage of allowing different genotypes to process and present the epitopes appropriate for their MHC polymorphisms.

What is the mechanism of tolerance? There can be at least two possible, nonmutually exclusive mechanisms: anergy as a result of antigen presentation by B cells in the absence of costimulation (22, 23) or active suppression by regulatory cytokines (5). Whereas lack of costimulatory signals might explain the protective effect of transduced bone marrow cells, it is difficult to invoke for LPS-stimulated B cells because LPS stimulation upregulates B7.1 and B7.2 expression. We do not know how long the LPS blasts remain activated in vivo, and indeed it is possible that expression of the costimulatory molecules is rapidly lost. Nevertheless, some reports indicate that even activated B-cells can present specific epitopes in a tolerogenic manner (6, 24, 25),

raising the possibility that additional mechanisms might be at play. We hypothesize that initial interaction of specific T lymphocytes with activated B cells presenting the uveitogenic epitope can lead to activation and upregulation of CTLA-4 on the T lymphocytes (26). Upon re-encounter of a B7-expressing activated B cell, CTLA-4 gives an "off" signal to the T lymphocyte (27). This possibility is supported by our recent data in the model antigen system of phage λ repressor, which suggested that tolerogenic peptide presentation by donor LPS blasts requires compatible class II MHC molecules for tolerance to occur and that anti-CTLA-4 treatment interferes under some circumstances with induction of tolerance (28). Studies are in progress to determine the nature of the tolerogenic APCs in these long-term LPS blast recipients.

Some immunoregulatory regimens are able to inhibit EAU by induction of regulatory cytokines (29). In the present study, this does not appear to be the case. Lack of an obvious Th1/Th2 shift in either cytokine patterns or Ig isotypes and an apparently unchanged TGF- β response suggest that immune deviation, as it is currently understood, is not playing an important role in the protection. The protection was not transferable, further supporting the notion that induction of regulatory cells is not a primary mechanism. Interestingly, whereas both IL-10 and IFN- γ production to antigen were depressed in treated recipients, there was no global suppression of cytokine synthesis. This, together with the partial inhibition of DTH and lymphocyte proliferation, contrasting with the impressive protection from disease, suggests that antigen-specific clones are not deleted *per se* by the tolerogenic treatment. Partial unresponsiveness, which could reflect tolerance only of high-affinity clones, is therefore sufficient to achieve clinical success in this system. The interpretation that it is the high-affinity clones that are preferentially tolerized by this regimen is also in line with the marked dose-response shift in the proliferation assay. In this context it is interesting to point out a similarity to IRBP-transgenic mice, which express a portion of IRBP containing the 161-180 epitope extraocularly under control of a class II promoter. These mice, which are highly refractory to induction of EAU, also show a 20-fold dose-response shift in antigen-specific proliferation, rather than complete unresponsiveness (30). A thorough dissection of the relative contributions of various possible mechanisms will be made feasible by the development of transgenic mice expressing uveitogenic T-cell receptors.

In summary, we have demonstrated that gene therapy with autologous cells transduced with a retroviral construct composed of a uveitogenic epitope fused with an isologous IgG molecule can prevent as well as reverse EAU. The effectiveness of this therapy in preimmune as well as in naive recipients opens the possibility of using this approach in a clinical situation when the patient has a preexisting repertoire of lymphocytes primed to an autologous antigen.

Acknowledgments

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EXHIBIT E

Induction of hyporesponsiveness to intact foreign protein via retroviral-mediated gene expression: The IgG scaffold is important for induction and maintenance of immune hyporesponsiveness

(gene therapy/immune self-tolerance/retroviral vector/antigen presentation)

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ABSTRACT IgG molecules can be highly tolerogenic carriers for associated antigens. Previously, we reported that recipients of bone marrow or lipopolysaccharide-stimulated B-cell blasts, both of which were retrovirally gene-transferred with an immunodominant peptide in-frame with the variable region of a murine IgG heavy chain, were rendered profoundly unresponsive to that epitope. To further investigate whether tolerance to larger molecules can be achieved via this approach and whether the IgG scaffold is important for induction and maintenance of immunological tolerance, we engineered two retroviral constructs encoding the $\text{cI } \lambda$ repressor (MBAE-1-102 and MBAE-1-102-IgG) for gene transfer. Our results show that recipients of bone marrow or peripheral B cells, transduced with the MBAE-1-102-IgG recombinant, are hyporesponsive to p1-102. In addition, the self-IgG scaffold enhanced the induction and maintenance of such an immune hyporesponsiveness. Thus, our studies demonstrate that *in vivo*-expressed IgG heavy chain fusion protein can be processed and presented on the appropriate MHC class II, resulting in hyporesponsiveness to that antigen and offering an additional therapeutic approach to autoimmune diseases.

Individuals normally develop tolerance to self-constituents during the development of the immune system. Tolerance induction, however, is a lifelong process and also must occur extrathymically (1). Moreover, the maintenance of this unresponsive state requires the persistence of antigen and continued induction in adults (2). The failure to discriminate between immunological self and nonself components leads to the clinical manifestations of autoimmunity. A number of experimental procedures have been proposed to induce tolerance to autoantigens and therefore to prevent and/or reverse autoimmune diseases (3–5), although tolerance induction and maintenance in mature animals has proven difficult. Hence, novel methods need to be developed to promote tolerance induction in immunocompetent adults and to express the tolerogen in multipotential hematopoietic compartments for persistence of tolerogen and long-term maintenance of tolerance.

Peptide fragments of multideterminant antigens can be divided into three main groups: dominant, subdominant, and cryptic epitopes (6–8). An immunodominant epitope is a peptide fragment specifically processed by antigen-presenting cells from a larger, multideterminant antigen and varies indi-

vidually as a function of its MHC. Such an epitope is capable of binding to the MHC molecule, and this peptide/MHC complex then is recognized by the T-cell repertoire (6, 7). Subdominant epitopes are the determinants that can stimulate native protein primed cells to proliferate, but less than dominant epitopes or the whole protein (7, 8). In contrast, cryptic determinants are rarely revealed during antigen processing and therefore fail to activate T cells when the native antigen is used as immunogen (7, 8). However, these hidden determinants might play a role in pathogenic autoimmune responses. One of the pathophysiological mechanisms that may explain the unveiling of these minor epitopes is determinant spreading (9, 10).

Immunoglobulins have been used as carriers to induce T- and B-cell tolerance to their own and associated epitopes, with isologous IgG carriers being the most efficacious (11, 12). Recently, Zambidis *et al.* (13) demonstrated the specific tolerogenic properties in adult BALB/c mice of a chimeric molecule consisting of residues 12–26 of the $\text{cI } \lambda$ repressor protein (p1-102) fused to the N terminus of a murine IgG H chain. In addition, animals receiving retrovirally encoded 12–26-IgG were shown to be profoundly unresponsive to the 12–26 peptide at both the humoral and cellular levels (14).

In cases where immunodominant epitopes have not yet been mapped, it would be desirable to fuse the entire protein to the IgG scaffold for tolerance induction. However, it is unclear whether a chimeric molecule consisting of full-length protein would be efficiently processed and presented and in turn tolerize as effectively as selected epitopes. Furthermore, it is unclear whether the IgG scaffold is essential for induction and maintenance of tolerance.

To address these questions, we established two MBAE retroviral constructs encoding either p1-102 or p1-102-IgG heavy chain. The constructs were used to transduce B cells or bone marrow (BM) cells, which were adoptively transferred to syngeneic mice to test for tolerance. When challenged with p1-102, mice receiving either LPS B-cell blasts or BM transduced with the 1-102-IgG-encoding gene failed to respond as effectively as the mock controls to the major epitopes of p1-102 recognized by mice of these haplotypes. Moreover, compared with 1-102 alone, the 1-102-IgG fusion protein was more effective in the induction of hyporesponsiveness. The former induced only a transient form of hyporesponsiveness,

Abbreviations: BM, bone marrow; LPS, lipopolysaccharide; HEL, hen egg lysozyme; NIP, nitroiodophenyl.

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suggesting that the IgG scaffold is important in the maintenance of tolerance. These results show that retrovirally mediated transfer of a gene encoding full-length protein fused to IgG is an effective approach for the purpose of inducing hyporesponsiveness to multiple epitopes.

MATERIALS AND METHODS

Mice. CB6 F₁ mice were purchased from The Jackson Laboratory at 6–8 weeks of age and housed in pathogen-free micro-isolator cages in our animal facility.

Retroviral Constructs Encoding λ p1–102 or p1–102-IgG and Virus Producer Cell Lines. A 320-bp DNA fragment encoding p1–102 was amplified by PCR (30 cycles: 94°C, 15 sec; 55°C, 15 sec; 72°C, 1.5 min) from pRB104 (a kind gift from Richard M. Breyer, Vanderbilt Medical Center, Nashville, TN). The 5' primer, GCG GTC GAC ATG AGC ACA AAA AAG AAA CC, contained a *Sall* restriction site and N-terminal sequences of 1–102; the 3' primer, CGC AAG CTT CTA CTA CTC ATA CTC ACT TCT AAG TGA, contained a *HindIII* restriction site and translational stop codon sequences. DNA polymerase fidelity was confirmed by subcloning the PCR product into pCRII vector (Invitrogen) and sequencing both DNA strands with universal cycle primer and reverse cycle primers (Amersham Life Science). The amplified p1–102 DNA fragment was cloned into *Sall*/*HindIII* restriction sites of the Moloney leukemia retroviral vector MBAE (14, 15).

For the p1–102-IgG construct, a 320-bp DNA fragment encoding p1–102 was PCR-amplified by using 5' and 3' primers containing a *NotI* restriction site and a *Sall* site, respectively. The p1–102 DNA subsequently was inserted into the V_H sequence of a murine IgG1 heavy chain between the 5' first framework region (FR1) and FR1 repeat. The resulting p1–102-IgG fragment, which included the leader sequence, V_H region inserted with p1–102, DJC region, and stop codon sequences, then was subcloned into MBAE downstream of a human β -actin promoter/enhancer (14, 15). As the original murine IgG1 heavy chain binds with high affinity to the nitro dophenyl (NIP) hapten when assembling with λ light chain, the recombinant 1–102-IgG fusion protein can be detected with a NIP-gelatin binding ELISA, although this is an underestimate of secreted IgG fusion protein (13, 14, 16).

Virus-producer cell lines (F5.19 and F12.7) were prepared by lipofection of ψ -2 packaging cell lines with p1–102-MBAE and p1–102-IgG-MBAE retroviral constructs, respectively, and were found to be helper virus free and to contain $\sim 10^5$ – 10^6 (usually $\geq 5 \times 10^5$) neomycin-resistant NIH 3T3 colony-forming unit/ml, by using methods as described (14, 15). Either ψ -2 parental cells or 52/139 virus-producer cell line was used as mock control. Virus-producer cell line 52/139 is a ψ -2 packaging cell line lipofected with MBAE retroviral construct encoding an irrelevant epitope in-frame with the murine IgG1 scaff ld (17).

Purification of 6 \times His-1–102 Protein and Synthesis of Antigenic Peptides. A recombinant 6 \times His-tagged 1–102 protein was engineered by subcloning the p1–102 DNA fragment into the pQE-31 vector (QIAGEN Expressionist Kit, QIAGEN, Valencia, CA) and transforming *Escherichia coli* M15 (pREP4) (QIAGEN Expressionist Kit). The p1–102 protein was prepared and purified by using a Ni-nitrilotriacetic acid column according to the manufacturer's instructions. Eluted p1–102 fractions were dialyzed against PBS (pH 7.2), filter-sterilized, and antigenically verified by ELISA and Western blot analysis (data not shown). Western blot analysis was performed by using the mAb B3.11, specific for the 12–26 epitope of p1–102 (13, 14).

The major antigenic peptides of p1–102 in H-2^d, H-2^b, and H-2^k mice, residues 12–26 (LEDARRLKAIYEKKK), residues 73–88 (VEEFSPSIAREIYEMY), and residues 55–69

(NALNAYNAALLAKIL), respectively (6, 18) were synthesized in the Molecular Biology Core of the Holland Laboratory by using a solid-phase method and were purified to $\geq 95\%$ homogeneity by HPLC.

Retroviral-Mediated Gene Transfer to BM and LPS-Stimulated B-Cell Blasts. Retroviral-mediated gene transfer into BM and bacterial LPS (*E. coli* 055:B5, Sigma)-stimulated splenic B cells has been described (14, 15, 19). Briefly, cells were cultured (3×10^6 /ml, 5 ml cultures) for 48 hr with irradiated (2,000 rad) F5.19/F12.7 or mock control packaging cell lines in the presence of 6 μ g/ml of polybrene and either 50 μ g/ml of LPS for B-cell blasts or 200 units/ml of IL-3, IL-6, and IL-7 (Genzyme) for BM. For adoptive transfer of BM, adult CB6 F₁ mice exposed to 400-rad irradiation were injected i.v. with 1 – 2×10^6 gene-transferred or mock-transduced BM cells. For adoptive transfer of LPS B-cell blasts, nonirradiated CB6 F₁ mice were injected with at least 1×10^7 transduced LPS blasts.

Immunologic Protocols. Ten days after receiving transduced LPS B-cell blasts or 6–8 weeks in the case of adoptive transfer of BM, the mice were s.c. immunized in one footpad and at the base of tail with 20 μ g of recombinant 6 \times His-p1–102 protein emulsified 1:1 in complete Freund's adjuvant (CFA) or with 20 μ g of hen egg lysozyme (HEL) in CFA as a specificity control. Two weeks later, mice were bled for the measurement of serum primary antibody responses. The mice then were either sacrificed and cellular immune responses in lymph nodes and spleen determined or boosted i.p. with 20 μ g of 6 \times His-p1–102 protein and 20 μ g of HEL in PBS. The secondary antibody responses were measured from sera collected 1 week after the boosting. Serum p1–102-specific or HEL-specific IgG responses were determined by ELISA by coating plates with 50 μ g/ml of synthetic peptide or 1 μ g/ml of HEL or p1–102. Antibody IgG titers were calculated as the dilution of test serum needed to reduce the signal to preimmune level. Splenic memory T cell responses were measured *in vitro* 1.5–5 months after secondary challenges by using [³H]thymidine incorporation, as described (14). IL-2 production (24-hr supernatants) was determined by either measuring CTLL-2 proliferation or ELISA by using Cytoscreen immunoassay kit (BioSource International, Camarillo, CA). Murine IL-4 and IFN- γ (48-hr supernatants) were determined by sandwich ELISA methods using the antibodies from PharMingen and recombinant cytokines as standards. Relative differences in cytokine levels at a given dose of antigen were used to estimate the degree of hyporesponsiveness.

Semiquantitative Genomic DNA PCR Analysis and Reverse Transcription-PCR (RT-PCR). Genomic DNA and total RNA were isolated from BM and spleen tissues from recipient mice and the p1–102 sequence was amplified from total RNA after first-strand synthesis (RT-PCR) or from genomic DNA. The amplified products were verified by Southern blot using p1–102 DNA as probe, as described (14, 20). The endogenous murine β -actin fragment was coamplified with 5' primer, AAG AGA GGT ATC CTG ACC CTG, and 3' primer, ATC CAC ATC TGC TGG AAG GTG. Genomic DNA and RNA from A20.2J cells transfected with 1–102/1–102-IgG genes were used for semiquantitation (20).

RESULTS

Recipient Mice of BM and LPS B-Cell Blasts Transduced with MBAE-1–102-IgG Are Hyporesponsive to p1–102 and Its Dominant Epitopes in H-2^{bmd}. Previously (14), we demonstrated that LPS B-cell blasts and BM cells transduced with the immunodominant epitope of p1–102 in-frame with an IgG heavy chain resulted in specific tolerance to that epitope. Immune responses to p1–102 in various MHC backgrounds have been well documented (6, 18). That is, BALB/c (H-2^d) and C57BL/6 (H-2^b) mice predominantly recognize epitopes

contained in residues 12–26 and 73–88, respectively (6, 18), whereas F₁ offspring between these two strains, CB6 F₁ mice (H-2^{bxd}), recognize both 12–26 and 73–88 (Y.K. and D.W.S., unpublished work). Consequently, we wanted to test whether the full-length p1–102 molecule fused to an IgG heavy chain could be processed in a tolerogenic manner and if the responses to its major epitopes also would be reduced.

To test the tolerogenicity of 1–102-IgG-transduced LPS B-cell blasts, purified splenic B cells from CB6 F₁ mice were stimulated with LPS, cocultured with F12.7 packaging cells, and subsequently injected i.v. into immunocompetent syngeneic CB6 F₁ mice. The efficacy of gene transduction was confirmed by detection of NIP hapten-specific p1–102-IgG recombinant protein in the sera of recipient mice, as well as by measurement of p1–102 mRNA expression in the spleen samples (data not shown; see refs. 14 and 20 and later in Fig. 7). As shown in Fig. 1, injection of 1–102-IgG-gene-transferred LPS blasts resulted in specific cellular hyporesponsiveness to the dominant epitopes of p1–102 in a H-2^{bxd} background (p12–26 and p73–88). Moreover, all of the mice produced comparable anti-HEL IgG titers (data not shown), whereas there was 3- to 5-fold reduction in the antibody response to p1–102 in recipients of 1–102-IgG-transfected blasts ($P < 0.05$, Fig. 2, Upper). Specific antibody responses to H-2^d and H-2^b immunodominant epitopes (12–26 and 73–88, respectively) were also significantly lower in the 1–102-IgG recipient mice than in mock controls ($P < 0.05$, Fig. 2, Lower).

In addition, the cytokine production in response to recall antigen (p1–102) was determined from *in vitro* cell culture supernatants. As shown in Fig. 3, a more than 3-fold reduction of IL-2 (based on relative amounts of cytokine produced at a given dose of antigen) and a less dramatic but significant inhibition of IFN- γ and IL-4 were demonstrated in recipient mice of MBAE-1–102-IgG-transduced LPS B-cell blasts, suggesting that this treatment also induced hyporesponsiveness in both CD4 Th1 and Th2 cell compartments.

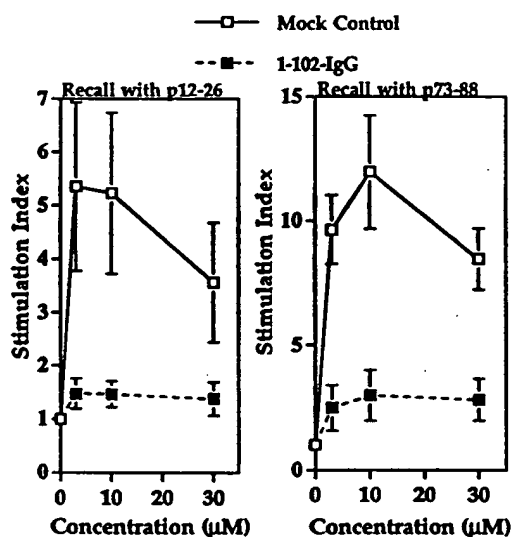


FIG. 1. T-cell responses to the dominant epitopes of p1–102 in CB6 F₁ recipients of 1–102-IgG-transduced LPS B-cell blasts. CB6 F₁ mice were injected with mock-transduced or F12.7 gene-transduced LPS B-cell blasts. Mice then were immunized with p1–102 and HEL. Two weeks later, lymph nodes were restimulated with dilutions of synthetic peptides, representing H-2^d (p12–26, Left) or H-2^b (p73–88, Right) immunodominant epitopes and pulsed with [³H]thymidine. The incorporated [³H]thymidine was detected by using a direct beta counter. One set of representative experiments is shown, with 3–5 mice per group. All mice respond equally to HEL. Stimulation index refers to (cpm/background cpm). Background cpm vary from 150 to 300 in this set of experiments. Data are presented as mean \pm SE.

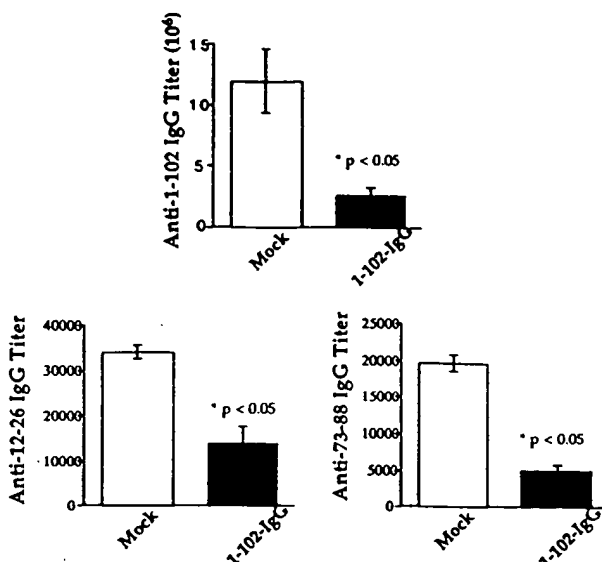


FIG. 2. Antibody responses to p1–102 and epitopes in CB6 F₁ recipients of 1–102-IgG-transduced B-cell blasts. CB6 F₁ mice were pretreated as described in Fig. 1. Mice were primed and boosted with p1–102 and HEL. Antibody IgG immune response was measured by ELISA. One set of representative experiments is shown, with 3–4 mice per group. All mice respond equally to HEL.

To develop a more effective tolerogenic protocol, we generated BM chimeras in sublethally irradiated (400 R) CB6 F₁ mice by infusing syngeneic donor BM cells that had been retrovirally transduced with 1–102-IgG heavy chain. The BM chimeras then were immunized with p1–102 as well as HEL in complete Freund's adjuvant. Because genetically modified BM possesses the potential to tolerize the reconstituting immune

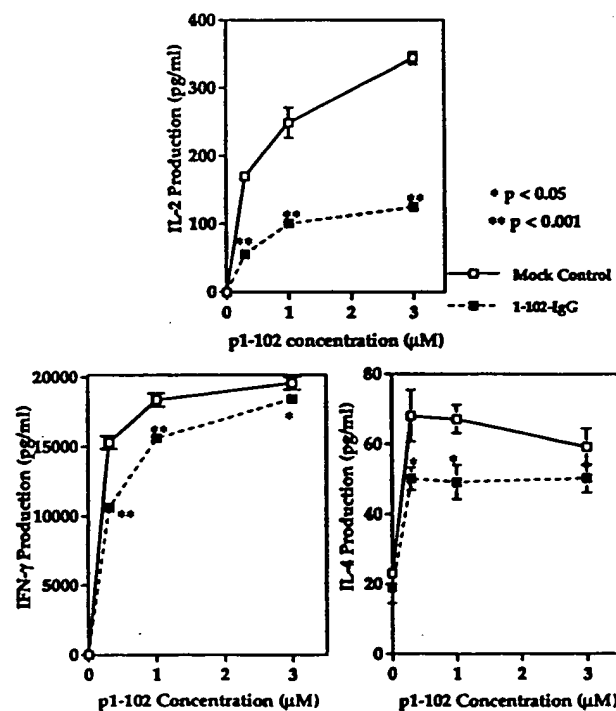


FIG. 3. Cytokine responses to p1–102 in CB6 F₁ recipients of 1–102-IgG-transduced B-cell blasts. CB6 F₁ mice were pretreated and immunized as described in Fig. 1. One set of representative experiments is shown, with 3–5 mice per group. Data are presented as mean \pm SE.

repertoire, we expected that recipient mice would have a more profound form of tolerance than seen in animals receiving the gene-transduced LPS blasts (Fig. 4). The pattern of hyporesponsiveness between these two protocols is similar. Importantly, more than a one log reduction of anti-p1-102 IgG titers was found in recipient mice of MBAE-1-102-IgG-transduced BM cells ($P < 0.05$) and unresponsiveness to its multiple epitopes also was observed, including a reduction in the antibody response to the 55-69 epitope, which is regarded as a minor determinant in H-2^{bxd} MHC haplotype (Fig. 4). This result agrees with the notion that tolerized mice are unresponsive both to the major and minor epitopes. T cell *in vitro* proliferation to the dominant epitopes of p1-102 was significantly reduced (data not shown), and the cytokine responses to p1-102 also were significantly suppressed in the recipient mice of 1-102-IgG transduced BM (see Fig. 6).

The IgG Scaffold Favors Long-Lasting Hyporesponsiveness at Both Cellular and Humoral Levels. To address the question of whether the self IgG scaffold is important in the induction and maintenance of such a hyporesponsiveness, we constructed a MBAE retroviral vector encoding the 1-102 DNA sequence without the self IgG scaffold. The MBAE-1-102 retroviral vector was constructed by insertion of a 1-102 DNA fragment downstream of the β -actin promoter/enhancer in the absence of a leader/secretion signal sequence, whereas the MBAE-1-102-IgG used above was similarly constructed but with an IgG heavy chain leader sequence. Nonetheless, only 1/3-1/2 of the latter recipients had a detectable level of NIP-specific IgG circulating in the serum (data not shown), which may reflect either the insensitivity of this assay to detect minimal amounts of secreted 1-102-IgG or an underestimate because the 1-102-IgG heavy chain binds to NIP only when it assembles with a λ light chain.

Groups of CB6 F₁ mice were injected as above with either LPS B-cell blasts or syngeneic BM cells that had been retrovirally transduced with 1-102 or 1-102-IgG heavy chain. Subsequently, recipients were immunized and boosted with p1-102 and HEL, and antibody responses were measured before and after the boosting. Interestingly, although injection

of 1-102 gene-transferred LPS and BM could induce a state of hyporesponsiveness to p1-102 primary immunization, this state was not long-lasting, based on the humoral responses to secondary challenge (Fig. 5 A and B). In contrast, 1-102-IgG gene-transmitted CB6 F₁ recipients maintained a state of stable and long-lasting hyporesponsiveness at the humoral levels. Furthermore, as shown in Fig. 6, the IgG scaffold favors a long-lasting immune hyporesponsiveness at the T-cell level as well. The splenic memory T-cell responses were measured 1.5-5 months after secondary challenge. Compared with p1-102 recipients, the p1-102-IgG recipients demonstrated a much more significant inhibition in T cell *in vitro* proliferation and cytokine production in response to recall antigens (Fig. 6). These results strongly suggest that the self IgG plays a crucial role in induction and maintenance of immunological tolerance. The difference between these groups cannot be attributed to gene expression, because as shown in Fig. 7 there were comparable levels of RNA expression in both groups. Moreover, long-term proviral DNA integration and p1-102 expression can be seen in BM and spleen tissues at 8 months after BM gene transfer (Y.K. and D.W.S., unpublished data).

DISCUSSION

The goal of the present study was to examine whether a gene therapy approach could be used to induce tolerance or hyporesponsiveness to multiple epitopes in a full-length protein. By using p1-102 as a model foreign antigen, we demonstrate herein that both LPS B-cell blasts and BM cells transduced with a retroviral recombinant encoding p1-102 in-frame with murine γ heavy chain are tolerogenic, resulting in specific hyporesponsiveness to p1-102 and its dominant epitopes at both cellular and humoral levels.

Residues 55-69 of p1-102 are recognized by T cells in I-A^k mice but may be considered a minor or cryptic epitope in I-A^{bxd} animals (6, 18). Cryptic epitopes usually are not processed and presented by antigen-presenting cells from the native protein (7, 8). If these cryptic determinants become visible, they can trigger pathogenic autoimmune responses (9, 10, 21). Therefore, it is desirable for a potential therapeutic approach to be able to turn off the responses not only against the major epitopes but also against the minor epitopes. Our results suggest this may be possible. Antibody titers to p12-26, p73-88, and p55-69 were reduced in CB6 F₁ recipient mice of adoptive BM transfer.

It is noteworthy that the responses against p1-102 in the recipient mice of BM and LPS B blast transduced with MBAE-1-102-IgG are only partially reduced. This specific hyporesponsiveness, however, might be sufficient to eliminate the pathophysiological processes in an autoimmune response. By using a similar approach, with a major pathogenic epitope of interphotoreceptor retinoid binding protein, Agarwal *et al.* (17) demonstrated that this form of gene therapy induced epitope-specific protection in experimental autoimmune uveitis. Interestingly, many parameters of immune responsiveness, such as T-cell proliferation and *in vitro* IL-2 and IFN- γ production, were only partially suppressed (17).

Note that the 6 \times His-tagged p1-102 used in our current study was prepared from transformed *E. coli* M15 bacteria. Because it is not uncommon for such a preparation to be contaminated with trace, highly immunogenic bacterial products, the effect of gene therapy for a particular protein (p1-102) may be underestimated with this immunogen. In contrast, this is not the case with purified peptides as targets (14).

A potential clinical therapy depends on its capability of reversing an ongoing autoimmune response. Although not yet tested with our current p1-102-IgG construct, results with 12-26-IgG and interphotoreceptor retinoid binding protein-IgG clearly demonstrate that such an approach could reverse

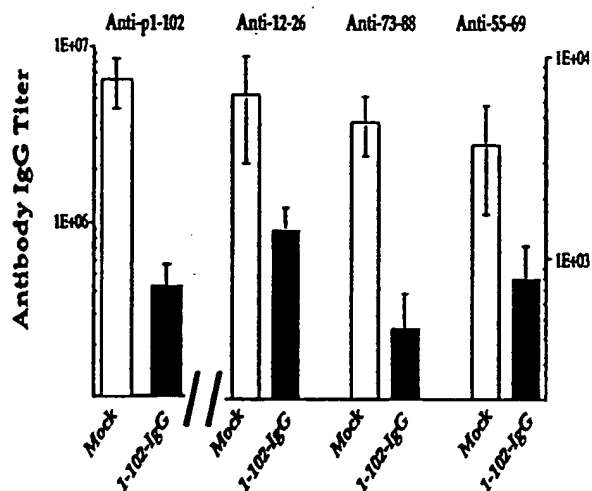


FIG. 4. Humoral hyporesponsiveness to p1-102 in CB6 F₁ mice receiving 1-102-IgG-transduced BM. CB6 F₁ mice were irradiated with 400 rad and injected with mock-transduced or F12.7 gene-transduced BM cells. Mice were primed and boosted with p1-102 and HEL. Left Y ordinate represents the IgG titer against p1-102, and the right Y ordinate represents the titers against epitopes. Antibody IgG titers against p1-102 and p73-88 in recipient mice of F12.7 transduced BM (filled columns) are significantly lower than those in mock controls (empty columns) ($P < 0.05$). Note that antibody against the minor epitope (p55-69) also is reduced in the recipient mice of F12.7-transduced BM.

A: LPS B Blast Gene Transfer

B: Bone Marrow Gene Transfer

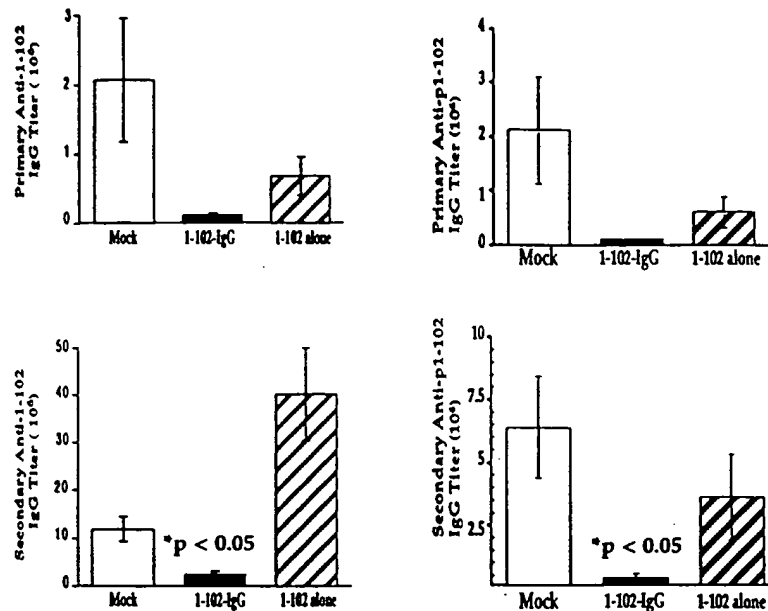


FIG. 5. Efficacy in tolerance induction and maintenance between 1-102-IgG and 1-102 gene transfer. (A) LPS blast recipients. (B) BM recipients. (Upper) Primary immune response. (Lower) Secondary immune response.

established immunity. Agarwal *et al.* (17) showed that multiple infusions of retrovirus-transduced B cells could protect primed recipients from experimental autoimmune uveitis. Though resting B cells from 12-26-IgG-expressing transgenic mice had no effect on an ongoing immune response, LPS-stimulated B-cell blasts from such transgenic mice induced tolerance in

BALB/c mice already primed for up to 3 months (16, 22). These data suggest a potential usefulness of our gene therapy in a clinical setting, e.g., in diabetes.

Self-tolerance is critical in the maintenance of homeostasis of the immune system and can occur during T- and B-cell maturation in the thymus and BM, or in the periphery, leading to central tolerance and peripheral tolerance, respectively (1, 23, 24). In our studies, recipient mice reconstituted by BM may recognize p1-102 as a self-antigen and induce both central and peripheral tolerance, whereas LPS B-blast recipients may induce tolerance only via peripheral mechanisms. Nevertheless, the use of LPS B-cell blasts transduced with a gene-encoding antigen has advantages over BM gene transfer by the relative simplicity of the approach and the lack of a requirement for myeloablation. We expect that repeated injections of gene-transduced LPS B-cell blasts at appropriate intervals would produce a much more profound state of unresponsiveness than a single injection, which is true with the experimental autoimmune uveitis animal model. Although no protection was observed with a single bolus of retrovirus-transduced LPS B blasts in the primed animals, three infusions at 2-day intervals significantly reversed the established experimental autoimmune uveitis (17).

The mechanism underlying tolerance induced by gene-transferred LPS B-cell blasts is not yet fully understood. By using transgenic mice expressing 12-26-IgG constitutively in B

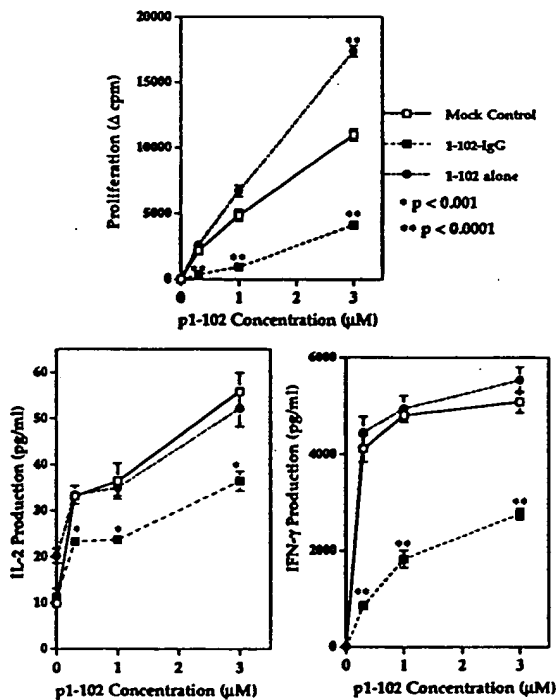


FIG. 6. Difference in memory T-cell responses between 1-102-IgG and 1-102 gene transfer. CB6 F₁ mice were pretreated, immunized, and boosted as described in Fig. 4. T-cell proliferation and cytokine production of splenic T cells was measured 1.5 months after immunization. One set of representative experiments is shown, with five mice per group. Data are presented as mean \pm SE.

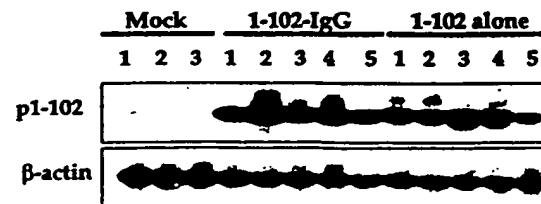


FIG. 7. Comparable levels of p1-102 mRNA expression between 1-102-IgG and 1-102. Spleen samples from the same recipients as in Fig. 6 were harvested (approximately 4 months after gene transfer), and the p1-102-specific mRNAs were measured by reverse transcription-PCR followed by Southern blotting using p1-102 as a probe.

cells, we did not observe any general defect in antigen-presenting cell capacity of these transgenic B cells (22). It is well known that productive T-cell activation requires at least two signals (25), as does B-cell activation. Although it is known (26, 27) that small, resting B cells are effective at inducing tolerance because of the lack of costimulatory molecules, this may be an oversimplification because LPS B-cell blasts are B7.1⁺ and B7.2^{high}.

Under appropriate circumstances, activated B-cell blasts may present specific epitopes in a tolerogenic manner to T cells (14, 16, 28). Gene-transduced LPS-activated B cells may serve as antigen-presenting cells, providing T cells with costimulatory signals and suboptimal TCR engagement and resulting in back-signaling to T cells to up-regulate CTLA-4 molecule, the negative regulatory costimulatory molecule on T cells (29). Hence the B7-CTLA-4-mediated negative signaling pathways on the transduced and activated LPS B-cell blasts may play a dominant role and, therefore, prevent T-cell activation and proliferation. Consistent with this hypothesis, our most recent data demonstrated that blocking B7-CTLA-4 interaction *in vivo* by i.v. injection of anti-CTLA-4 antibodies reversed hyporesponsiveness induction (30).

During the last decade, retroviruses have been used for the expression of different genes as a prelude for human gene therapy (31). Expression of the retrovirally transmitted genes could be detected for more than 4–8 months (Fig. 7 and ref. 15). The majority of virally encoded proteins in the cytosolic compartment are processed and presented by the infected cells as MHC class I-peptide complexes for interaction with CD8 T cells (32). However, in some cases, endogenously derived peptides also can be routed to endocytic class II MHC compartments (14, 33, 34). Our previous (14) and current studies demonstrate that retrovirally transduced foreign protein in-frame with γ heavy chain can induce MHC class II-restricted tolerance, manifested by hyporesponsiveness to that protein at both the CD4 T cell level and antibody production. This finding may be the result of the efficient nature of the Ig secretory pathway in targeting the endosomal MHC class II compartment. Because a fusion protein built on an IgG heavy chain scaffold would not be secreted unless assembled with light chains, B cells would have a selective advantage in antigen presentation of locally secreted molecules.

Finally, expression of p1–102-IgG mediates a more effective and longer-lasting hyporesponsiveness than p1–102 alone (Figs. 5 and 6). We cannot rule out the possibility that the difference seen may be the result of more efficient secretion of p1–102-IgG into the bloodstream than that of p1–102 alone. Thus, the latter still may induce efficient tolerance in the CD8⁺ T cell compartment whereas the former causes more effective CD4 T-cell tolerance. However, it also is noteworthy that the efficacy of tolerance induction need not correlate with levels of gene expression (14, 20).

In summary, the data presented in this report have implications for designing gene therapy strategies for modulating antigen-specific immunity. Tolerance or even hyporesponsiveness to certain foreign proteins as well as autoimmune antigens may be useful in numerous clinical situations. More importantly, the ability to express such a construct in BM allows one to create tolerogens and to have the host produce such fusion proteins for the induction and maintenance of unresponsiveness to disease-inducing epitopes.

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